Endothelin-1 (ET-1) has been reported to be expressed in human epidermis at both the gene and protein levels. ET-1 plays a pivotal role in ultraviolet B (UVB)-induced pigmentation due to its accentuated secretion after UVB irradiation and its function as a mitogen and as a melanogen for human melanocytes. We have recently found that endothelin-converting enzyme (ECE)-1α plays a constitutive role in the secretion of ET-1 by human keratinocytes and that an extract of Sanguisorba officinalis L. inhibits ECE activity in human endothelial cells, which predominantly express ECE-1α. In this report, to clarify the potential use of this botanical extract as a whitening agent, we examined whether this extract inhibits UVB-induced pigmentation in vivo. When this extract was applied to human keratinocytes after UVB irradiation, secretion of ET-1 by those cells was reduced, and this was accompanied by a concomitant increase in the secretion of inactive precursor Big endothelin-1.

When hairless mice were exposed to UVB light and were treated with the extract, it suppressed the induction of ET-1 in the UVB-irradiated epidermis. In the course of UVB-induced pigmentation of brownish guinea pig skin, this extract significantly diminished pigmentation in UVB-exposed areas. These findings indicate that ECE-1α in keratinocytes plays a pivotal role in the induction of pigmentation following UVB irradiation and that an extract of S. officinalis, which inhibits ET-1 production in human keratinocytes, is a good ingredient for a whitening agent.

Key words Sanguisorba officinalis L. extract; endothelin-converting enzyme-1α; endothelin-1; ultraviolet B

Endothelin-1 (ET-1), which was initially isolated and sequenced from cultured pig endothelial cells, is a potent vasoactive peptide consisting of 21 amino acid residues with two disulfide bridges. Vasodilatory smooth muscle cells locally regulate the function of underlying smooth muscle cells by producing ET-1 as well as nitric oxide in response to various stimuli. ET-1 also plays an important role in regulating cutaneous melanogenesis, especially in ultraviolet B (UVB)-induced pigmentation, since ET-1 production by human keratinocytes and by the epidermis increased after irradiation with UVB in a dose-dependent manner, and this was accompanied by the significant secretion of interleukin 1α. It was demonstrated that proliferation and differentiation of human melanocytes, and tyrosinase expression, are stimulated by ET derivatives via a receptor-mediated signal transduction pathway.

In the search for whitening agents that can suppress such ET-1 functions, we have found that extracts of Matricaria chamomilla L. and Althaea officinalis L. inhibit ET-1-induced intracellular calcium mobilization and proliferation of human melanocytes. When an extract of M. chamomilla was applied to human skin after UVB irradiation, pigmentation was significantly diminished. This extract has been actually used as a whitening agent in our products. To improve the inhibitory effects of our whitening products on ET-1 function and melanin synthesis, we have continued to look for new agents which inhibit ET-1 production in human keratinocytes, and this was accompanied by a concomitant increase in the secretion of inactive precursor Big endothelin-1.

We have recently revealed that ECE-1α plays a pivotal role in the secretion of ETs by human keratinocytes using an ECE-1α-specific immunoprecipitating antibody (Hachiya et al., manuscript in preparation).

Therefore, inhibition of ECE activity in human keratinocytes would be expected to suppress UVB-induced pigmentation. It was reported that ECE activity is inhibited by the metalloprotease inhibitor phosphoramidon, but not by thiorphan, a specific inhibitor of neutral endopeptidase 24.11 (NEP). However, phosphoramidon is not suitable for use as a cosmetic agent because of its low penetration of the skin and its expense. Therefore, we have tried to find an ECE inhibitor which is suitable for use as a cosmetic agent.

We have found that an extract of Sanguisorba officinalis L. inhibits ECE activity in human endothelial cells. S. officinalis is found in China, Korea, Japan, Siberia and Europe, and extracts of S. officinalis have been used as fungicides, astringents and hemostatics. In this report, to clarify the suitability of this botanical extract as a whitening agent, we examined whether it inhibits UVB-induced pigmentation in vivo.

**MATERIALS AND METHODS**

**An Extract of Sanguisorba officinalis L.** An extract of Sanguisorba officinalis L. was obtained as commercially available product. It was extracted from roots of S. officinalis with 50% EtOH at room temperature.

**Materials** Normal human endothelial cells, keratinocytes and E300 medium were obtained from Kyokutou (Tokyo, Japan). Big ET-1, ET-1, and ET enzyme-linked immunosorbent assay (ELISA) kits were purchased from Immuno-Biological Laboratories (Gunma, Japan). Serum-free keratinocyte medium (SFM), bovine pituitary extract (BPE), epidermal growth factor (EGF) and DMEM were purchased.

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from Gibco Laboratories (Grand Island, NY, U.S.A.). Other chemicals were of reagent grade.

**Cell Culture** Cells were cultured at 37 °C with 5% CO₂. Human endothelial cells and keratinocytes were maintained in E300 medium and modified SFM supplemented with 5 ng/ml EGF and 50 µg/ml BPE, respectively.

**Animals** Outbred hairless mice and brownish guinea pigs (tortoishell guinea pigs) ranging in age from 8 to 12 weeks and 12 to 20 weeks, respectively, at the beginning of the study, were used. The animals had free access to food and chlorinated water and were housed in individual cages. Prior to UVB irradiation, each guinea pig was shaved with electric clippers to remove long hair. The remaining stubble was removed with an electric shaver (Braun).

**Measurement of ECE Activity** These techniques were performed according to the method of Xu et al. Briefly, the reaction mixture for ECE assay (100 µl) contained 0.1 M sodium phosphate buffer (pH 6.8), 0.5 M NaCl, 0.1 µM human Big ET-1 (1—38), 0.25 to 1.0% (v/v) of the botanical extract and 2 µg of the enzyme fraction. To prepare the enzyme fraction, human endothelial cells were washed three times with phosphate buffered saline (PBS) and were harvested with a rubber policeman in 50 mM sodium phosphate buffer (pH 7.8). The cell suspension was sonicated for 5 min with a Bioruptor (Olympus) and centrifuged at 10000×g for 20 min at 4 °C. The supernatant was then further centrifuged at 100000×g for 60 min at 4 °C. The resulting precipitate was dissolved in 25 mM sodium phosphate buffer (pH 6.8) containing 0.1% Triton-X 100 and was sonicated. The reactions were preincubated at 37 °C for 15 min prior to the addition of Big ET-1. After 2 h, the enzyme reaction was terminated by adding 100 µl of 5 mM EDTA and the mixture was then directly assayed for mature ET-1 (1—21) using an ELISA. The ET-1 ELISA kit is a solid phase enzyme immunoassay which uses the multiple antibody sandwich principle. A human purified polyclonal antibody specific for human ET-1 was attached to 96-well microtiter plates. ET-1 present in standards or in samples to be tested was captured by the solid phase antibody. Horseradish peroxidase-labeled rabbit anti-human ET-1 IgG was added, which bound to multiple epitopes on ET-1 attached to the solid phase antibody. Levels of immunoreactive ET-1 were measured for absorbance at 450 nm by a Model 550 ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The amount of ET-1 in each reaction mixture was determined by comparing its absorbance to that of standards. The standard curve was linear from 78.1 to 5000 pg/ml.

**Irradiation with UVB Light** Cells were seeded in 6 well plates (Falcon, Franklin Lakes, NJ, U.S.A.) at a density of 5×10⁴—10⁵ cells/ml. After cultivation for 24—96 h, the culture medium was replaced with PBS. The cells were washed three times with PBS and then kept in PBS. They were irradiated with 20 mJ/cm² of UVB, at which dose most of the energy is emitted within the UVB range (295—315 nm) with a peak at 305 nm.

For in vivo testing, irradiation was performed on the dorsal skin of hairless mice. Irradiation test sites of three square centimeters each were chosen and delineated with an indelible marker to mark the sites for subsequent treatments. Half of the dorsal skin area was irradiated twice at a 24-h interval with a dose of 40 mJ/cm² of UVB light, which corresponds to about 1 minimal erythema dose in total. The other half of each dorsal skin area was masked and was not exposed to UVB light.

On the dorsal skin of brownish guinea pigs, irradiation test sites of one square centimeter were chosen in areas of comparable baseline pigmentation and were delineated with an indelible marker to mark the sites for subsequent treatments. The guinea pigs were irradiated twice at a 24-h interval with a dose of 288 mJ/cm² of UVB light.

**Measurement of ET-1 and Big ET-1 Concentrations** To measure ET levels after UVB irradiation by ELISA, cells were seeded in 6 well plates (Falcon) at a density of 5×10⁴—10⁵ cells/ml and were cultivated for 24 to 96 h. After UVB irradiation, the culture medium was aspirated and replaced with fresh medium, and the cells were cultured for another 48 h. The conditioned medium was then collected and quantified in 100 µl/well for ET-1 and Big ET-1 by ELISA, as described above.

To measure the amount of ET-1 in mouse epidermis after UVB irradiation, it was removed after incubation in 2 na NaBr at 37 °C for 30 min and was homogenized in 2 ml of extraction buffer containing 1 m acetic acid and 20 mM HCl with a HG 30 Homogenizer (Hitachi, Tokyo, Japan). The suspension was then applied to SEP-PAK LIGHT 18 column (Waters Corporation, Milford, MA, U.S.A.) and eluted with 2 ml of elution buffer containing 0.1% trifluoroacetic acid and 60% acetonitrile. The eluted samples were frozen and dried. After dissolving the samples in PBS containing 1% BSA and 0.05% Tween20, the samples were applied to human ET-1 ELISA plates and assayed as described above, since the mouse ET-1 peptide sequence is identical to that of humans.

**Western Blotting** ECE-1 expression in human keratinocytes was investigated using western blotting analysis. Proteins from Nonidet P-40/SDS-solubilized cells were separated on 7.5% SDS gels, then transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA, U.S.A.) and incubated with a purified polyclonal rabbit antibody specific for ECE-1α (at 1/1000 dilution). That antibody recognizes the N-terminus of ECE-1α protein specifically (Hachiya et al., manuscript in preparation). Subsequent visualization of antibody binding was carried out with Enhanced ChemiLuminescence (Amersham Corp., Arlington Heights, IL, U.S.A.) according to the manufacturer’s instructions.

**Measurement of Skin Color** Prior to the measurement of skin color, the backs of the guinea pigs were shaved with electric clippers to remove long hair, and the remaining stubble was removed with an electric shaver (Braun). The intensity of UVB-induced pigmentation was measured by a color difference meter (Nippon Denshoukndo Industries, Tokyo, Japan) and is expressed as delta L value.

**Statistics** Statistical analysis was carried out using the Student’s t test.

**RESULTS**

**Inhibitory Effect of the Extract of *S. officinalis* on ECE Activity** To clarify the inhibitory effect of the extract of *S. officinalis* on ECE activity, we used the particulate fraction of human endothelial cell as the enzyme fraction since human endothelial cells have high levels of ECE-1 activity and ex-
press predominantly the ECE-1α isoform, as do human keratinocytes. When the extract of S. officinalis was added to the reaction mixture, ECE activity was significantly reduced in a dose-dependent manner (Fig. 1), and was reduced to 7.8% at 1.0% (v/v) (0.03% (w/v)) compared with the control. This inhibitory effect was stronger than that of 1 mM (0.059% (w/v)) of phosphoramidon (Boehringer Mannheim, GmbH, Germany).

Inhibitory Effect of the Extract of S. officinalis on ET-1 Levels in Mouse Epidermis

Prior to examining whether ET-1 levels in mouse epidermis is inhibited by this extract, we confirmed the amount of withdrawal of ET-1 after passing through the SEP-PAK LIGHT 18 column and eluted. After frozen and dried, the samples were dissolved and assayed in ELISA as described under “Materials and Methods.” The values represent means ± S.D. from three independent experiments.

Levels in Mouse Epidermis

Prior to examining whether ET-1 levels in mouse epidermis is inhibited by this extract, we confirmed the amount of withdrawal of ET-1 after passing through the SEP-PAK LIGHT 18 column and determined the time course of ET-1 expression during up-regulation in mouse epidermis after UVB irradiation. ELISA revealed that almost all amount of ET-1 after passing through the column were withdrawn (Fig. 4). Followed to confirmation of the amount of withdrawal of ET-1, when the samples extracted from mouse epidermis were applied to ELISA, ET-1 levels decreased 2 to 4 d after the first UVB irradiation but was up-regulated 6—10 d after that (Table 1). It would be easier to assess the inhibitory effect of the extract at the day when ET-
that respond well to several stimuli, including UVB light, guinea pigs have functional melanocytes in their epidermis. To examine whether the inhibition of ECE activity and as % of control. UVB-Induced Pigmentation in Brownish Guinea Pig Skin

ET-1 production was diminished in both systems. Additionally, application of that extract inhibited UVB-induced pigmentation in brownish guinea pig skin compared with the vehicle control. These findings indicate that ECE-1α in keratinocytes plays an important role in the induction of pigmentation after UVB irradiation and that the extract of S. officinalis is thus a potent ingredient for a whitening agent. The identification of ingredients inhibiting ECE activity remains to be done, whereas tannin is well known to be a component in the extract of S. officinalis and have various physiological activities.

Table 1. Levels of ET-1 in Mouse Epidermis after UVB Irradiation

<table>
<thead>
<tr>
<th>Days after the first UVB irradiation</th>
<th>Levels of ET-1 (% of control)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>100.0±5.6</td>
</tr>
<tr>
<td>6</td>
<td>143.2±40.4</td>
</tr>
</tbody>
</table>

The amount of ET-1 in mouse epidermis were analyzed by ELISA, as described under “Materials and Methods” and are expressed as relative to the non-exposed area and as % of control.

Fig. 5. Inhibitory Effect of the Extract of S. officinalis on ET-1 Production in Hairless Mouse Epidermis after UVB Irradiation

Hairless mice were exposed twice to UVB light and the extract of S. officinalis (5.0%, v/v) and phosphoramidon (1 mM) were applied to the dorsal skin twice daily for 10 d, as detailed in “Materials and Methods.” Measurements of color difference were carried out by color difference meter on day 10. The values represent means±S.D. from four independent experiments. *p<0.05.

DISCUSSION

In UVB-induced pigmentation, three major processes, the proliferation of melanocytes, the synthesis and activation of tyrosinase, and the transfer of melanosomes to keratinocytes, are involved. As for melanocyte proliferation, it has been reported that ET-1, which is produced by keratinocytes and is up-regulated after UVB irradiation, acts as a mitogen for human melanocytes. In addition to its function as a mitogen, ET-1 has also been reported to stimulate melanogenesis in human melanocytes via increased transcription of tyrosinase. It has been established that ET is active only after immature Big ET is cleaved at Trp-21-Val/Ile-22 to produce the mature peptide, ET. This processing step is carried out by ECE1 and cleavage by ECE-1α plays an important role in ET-1 secretion by human keratinocytes (Hachiya et al., manuscript in preparation).

In this study, in an effort to develop a whitening agent, we have focused on the inhibition of ECE-1α in human keratinocytes and we have found a botanical extract which significantly inhibits its activity. When the extract of S. officinalis, which abolishes ECE activity in cell extracts, was applied to human keratinocytes and the skin of hairless mice after UVB irradiation, ET-1 production was diminished in both systems. Additionally, application of that extract inhibited UVB-induced pigmentation in brownish guinea pig skin compared with the vehicle control. These findings indicate that ECE-1α in keratinocytes plays an important role in the induction of pigmentation after UVB irradiation and that the extract of S. officinalis is thus a potent ingredient for a whitening agent. The identification of ingredients inhibiting ECE activity remains to be done, whereas tannin is well known to be a component in the extract of S. officinalis and have various physiological activities.

One important issue to be addressed in creating a whitening agent to suppress ET-1 activity is whether that is more effective than inhibiting tyrosinase activity in controlling UVB-induced pigmentation, since tyrosinase is the rate-limiting enzyme in melanin synthesis. To inhibit tyrosinase activity, an inhibitor would have to penetrate both the melanocyte and the melanosomal membranes since tyrosi-
nase acts within those organelles in melanocytes. \textsuperscript{19} However, an inhibitor of ECE-1α would only need to reach the keratinocyte membrane, where ECE-1α exists, to suppress ET-1 biosynthesis, which would then result in the inhibition of ET-1-induced melanocyte proliferation. Furthermore, since ET-1 stimulates not only melanocyte proliferation, but also tyrosinase expression in human melanocytes, \textsuperscript{41} the extract of \textit{S. officinalis}, which does not inhibit tyrosinase activity itself, would have a dual action and would be more effective than a tyrosinase inhibitor in whitening human skin.

It has been demonstrated that ET-1 is also related to levels of constitutive pigmentation in addition to its role in UVB-induced pigmentation. For example, it has been suggested that ET-1 plays an important role in the hyperpigmentation seen in seborrhoeic keratosis (SK). \textsuperscript{20} SK is a common benign tumor with accentuated epidermal pigmentation. \textsuperscript{21} Hydroquinone is a reagent used to treat hyperpigmentary disorders, but it has been reported to cause irritation and dermatitis under certain conditions. \textsuperscript{22,23} If an ET-1 inhibiting agent, such as the extract of \textit{S. officinalis}, is applied to SK skin, it might only suppress the excess amount of ET-1 and might elicit no side effects. Therefore, the extract of \textit{S. officinalis} might also be effective in whitening other conditions of hyperpigmentation stimulated by ET-1.

Recently, we have found that production of stem cell factor (SCF), which is expressed in a membrane-bound form on the plasma membrane of keratinocytes, is stimulated in the early phase of UVB-induced pigmentation and acts as a mitogen and as a melanogen for human melanocytes (Hachiya \textit{et al.}, manuscript in preparation). Stimulation of ET-1 secretion by keratinocytes occurs in a later phase, and the secreted ET-1 may synergistically activate melanocyte function, probably through cross-talk in signaling pathways with SCF. \textsuperscript{24} Recent evidence suggests that there is a complex network in the epidermis for secreting and responding to autocrine and paracrine cytokines by keratinocytes and melanocytes, respectively. \textsuperscript{7,25—27} This is effected via corresponding receptors which are also modulated in their expression by various cytokines and there is a cross-talk in signaling between cytokines to support the accentuated activation of melanogenesis in melanocytes. In the near future, these cytokines and receptors will be targeted in a new generation of whitening agents.

In conclusion, these findings suggest that an extract of \textit{S. officinalis}, which inhibits ET-1 production in human keratinocytes, is a potent ingredient for a whitening agent.

REFERENCES